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12b. DISTRIBUTION CODE**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**

The development of estrogen receptor (ER) negative breast cancer is associated with development of alternate growth regulatory pathways. Three in vitro models of ER+ and ER- breast cancer were evaluated for expression and activation of MAP kinase and p70/S6 kinase as possible growth regulators in ER- breast cancer. Despite decreased activity of MAP kinase in ER- cells, growth of these cells is entirely dependent on signaling through MAP kinase as determined by analyzing growth in the presence of U0126, A MAP kinase inhibitor. Even though activity of p70/S6 kinase is maintained in ER- cells, growth is not affected by inhibition of p70/S6 kinase. These results suggest that signaling through the Ras/Raf/MAP kinase pathway is critical for the growth of ER- breast cancer cells.

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Introduction

Progression of breast cancer from an estrogen-dependent, ER positive status to an estrogen-independent, ER negative status is the focus of much current research.

Observations made both from *in vitro* models and clinical research indicates that upregulation of growth factor signaling pathways is correlated with the progression to estrogen independence and loss of estrogen receptor expression. Increased levels of MEK and Raf-1 as well as increased MAP kinase activity has been observed in breast cancers compared to normal breast tissue, indicating that hyperactivation of growth signaling pathways both at the cell surface and intracellularly is an important factor in breast cancer progression ^{1,2}. Increased expression of both the erbB2 and EGF receptors is observed in ER- breast cancer ³⁻⁶. *In vitro* models where these growth factor receptors have been over-expressed in the ER+ MCF-7 cell line suggest that increased signaling through growth factor receptors is an important step in the path to estrogen independence ^{7,8}. More recently, increased MAP kinase activity has been shown to contribute to the loss of ER- expression *in vitro* ^{9,10}.

The T47D:A18, MCF-7 and ZR-75-1 cell lines along with their ER negative counterparts, all derived by selecting for cell growth in the absence of estrogen, provide models for further examining the status of growth factor signaling pathways in ER- breast cancer. Preliminary data suggests that the ER- 2W, LCC3 and C4-12 cell lines have decreased MAP kinase activity compared to the parental ER + cell lines but maintain MAP kinase expression at levels similar to the parental cell lines. This observation indicates these cells may not be dependent on the MAP kinase pathway for growth. Further evaluation of these cell lines using western blot analysis has revealed that both

A18 and 2W cells have increased levels of p70/S6 kinase activity. p70/S6 kinase is a component of the PI-3-kinase pathway which is activated by ser/thr phosphorylation^{11,12}. Both Akt and phosphatidyl inositide dependent kinase I (PDKI) have been shown to mediate activation of p70/S6 kinase^{12,13}. Activation of p70/S6 kinase facilitates phosphorylation of the ribosomal S6 subunit and subsequently protein synthesis^{14,15}. p70/S6 kinase is primarily activated during the G0/G1 and G1/S phase transition, suggesting a role in cell cycle progression¹⁶. Additionally, a recent analysis has demonstrated gene amplification in 59% of breast tumors examined and this amplification was associated with a poorer prognosis¹⁷. While no correlation with ER status was observed, this observation suggests that S6 kinase may play an important role in breast cancer progression¹⁷. Expression and activity of S6 kinase in breast cancer contributes to breast cancer progression and represents an alternative growth pathway by which breast cancer cells can achieve estrogen independence.

Results

Alternate Signaling Pathways MAP kinase activity was assessed by western blot using phospho-specific antibodies that detect the activated form of MAP kinase (Figure 1, lower panel). MAP kinase expression was maintained at equal levels in all cell lines analyzed (Figure 1, upper panel) but total MAP kinase activity was decreased in the ER- cell lines, T47D:C4:2W, LCC3 and ECMCF-7. The decreased MAP kinase activity observed in the ER- breast cancer cell lines studied here suggested increased dependence on alternate growth regulatory pathways. A second major signaling pathway that receives signals from receptor tyrosine kinases is the PI-3 kinase pathway. While the activity of this pathway has been traditionally associated with cell

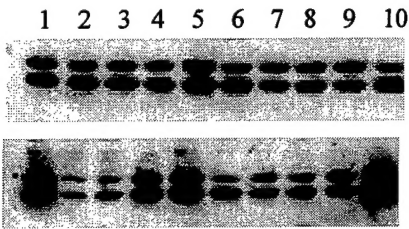


Figure 1 MAP kinase activity is decreased in ER- breast cancer cells.
1)T47D:A18 2)T47D:C4:2W
3)T47D:C4:9W 4)T47D:C4:10W
5)ZR-75 6) LCC3 7) WWMCF-7
8) ECMCF-7 9) MCF-7 10)MDA-231

survival, elements of the pathway, such as p70/S6 kinase can impact growth regulation and cross-talk between the PI-3 kinase and MAP kinase pathway has been demonstrated in several cell types, including breast cancer cells. The PI-3 kinase pathway was assessed by western blotting for expression and activation of P70/S6 kinase in each model.

p70/S6 kinase is phosphorylated on multiple residues by a number of kinases, including Akt, PDK-1 and mTOR. Expression of p7-/S6 kinase was assessed by western blot analysis (Figure 1, upper panel). Activation of p70/S6 kinase was determined by phospho-specific western blotting with antibodies against phospho-Thr389, a

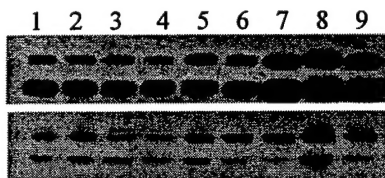


Figure 2 p70/S6 kinase activity is decreased in ER- breast cancer cells.
1)T47D:A18 2)T47D:C4:2W
3)T47D:C4:9W 4)T47D:C4:10W
5)ZR-75 6) LCC3 7) WWMCF-7
8) ECMCF-7 9) MCF-7

residue necessary for activation of kinase activity (Figure 2, lower panel). Expression of p70/S6 kinase was maintained in both the T47D and ZR-75 models (Figure 2, upper panel). Increased expression was observed in ER- ECMCF-7 cells compared to the parental ER+ MCF-7 cell line (Figure 2, upper panel). The ER+ T47D:A18 cells and ER- 2W cells

had similar amounts of activated p70/S6 kinase (Figure 1, lower panel) . A slight decrease in activated p70/S6 kinase was observed in ER- LCC3 cells compared to the parental ER+ ZR-75 cell line (Figure 2, lower panel). Finally, in the MCF-7 model, ER- ECMCF-7 cells demonstrated an increase in activity (Figure 2, lower panel) but these cells also had increased expression so that there was no relative change in activity between the two cell lines in this model.

The impact of p70/S6 kinase activity on growth was determined by evaluating proliferation in the presence of rapamycin. Rapamycin is a specific inhibitor of mTOR, the mammalian target of rapamycin, which participates in the activation of p70/S6 kinase by mediating phosphorylation of a Thr389, shown to be necessary for full activation of kinase activity. Treatment of T47D cells with 200nM rapamycin for 1 or 24 hours results in a complete loss of T389 phosphorylation (Fig inset). Growth in the presence of 200nM rapamycin is inhibited in the ER+ T47D:A18 cell line, but not the ER- 2W clone. Similar results were observed in the ZR-75 model, ER+ ZR-75 cells were growth inhibited in the presence of rapamycin while the ER-clone, LCC3, maintained normal growth rates. The MCF-7 model differed from the T47D and ZR-75 model since growth of both the ER+ MCF-7 cells and ER- ECMCF-7 cells was inhibited by rapamycin. The results suggest that despite the presence of active p70/S6 kinase in ER- 2W and LCC3, these cells are not dependent on p70/S6 kinase for growth. Therefore, it was important to determine what the critical growth pathway in these cells was.

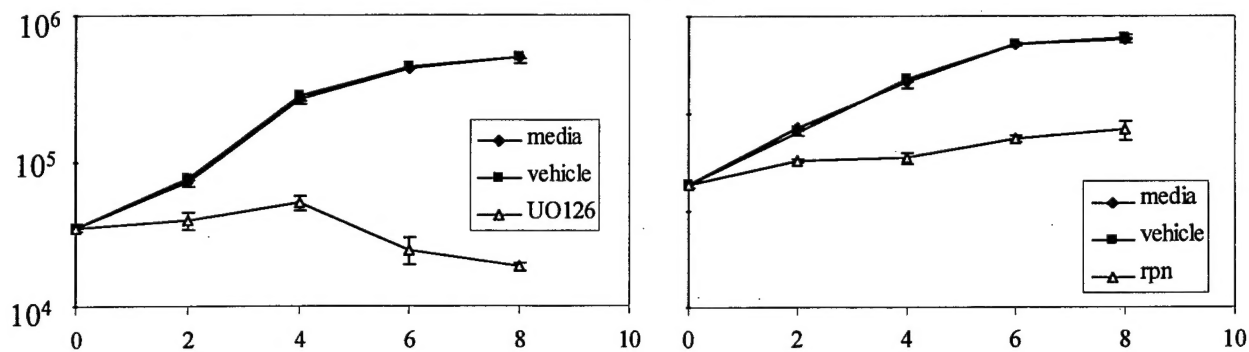
Similar experiments were performed in the presence of UO126, a specific inhibitor of MEK1/2, the upstream activator of MAP kinase. Western blot analysis demonstrates that MAP kinase activity in T47D and MCF-7 cells is abrogated by treatment with 10uM UO126 for 24 hours (Fig). MAP kinase activity in ZR-75 cells is abrogated by treatment with 25uM UO126 for 24 hours (Fig). Growth was inhibited by UO126 treatment in all of the cell lines examined, regardless of the ER status (fig), demonstrating that despite the decreased MAP kinase activity observed in the ER- cell lines, this pathway still remains the critical growth pathway in ER- breast cancer cells.

Conclusions

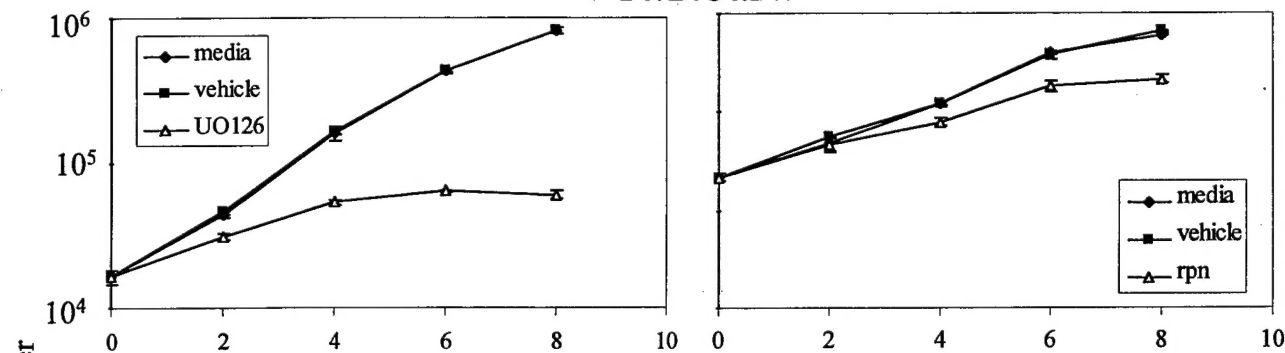
The results presented here demonstrate changes in the activity of growth regulatory pathways in ER- breast cancer cells. Multiple growth regulatory pathways are functional in ER+ cells as demonstrated by sensitivity to inhibitors of MAP kinase and P70/S6 kinase. In contrast, ER- cells exhibited changes in the activity of MAP kinase and in some cases, of p70/S6 kinase. Despite the decrease in activity of MAP kinase, the ER- cells were solely dependent on activation of this kinase for normal growth. The ER- cells were not dependent on activity of p70/S6 kinase for growth, as were the ER+ parental cell. This observation suggests that progression from an ER+ to an ER- phenotype involve alteration of signaling pathways within the cell. Signaling at the cell membrane will be analyzed by western analysis of ErbB family members and components of the Ras/Raf/MAP kinase pathway will be analyzed to determine if there is a general decrease in activity of this pathway in the ER- cells. Growth will also be analyzed in the presence of the inhibitor LY294002, a specific inhibitor of P-I-3 kinase to determine if growth of these cells is dependent on cross-talk between the P-I-3 kinase and MAP kinase pathways.

Figure 3

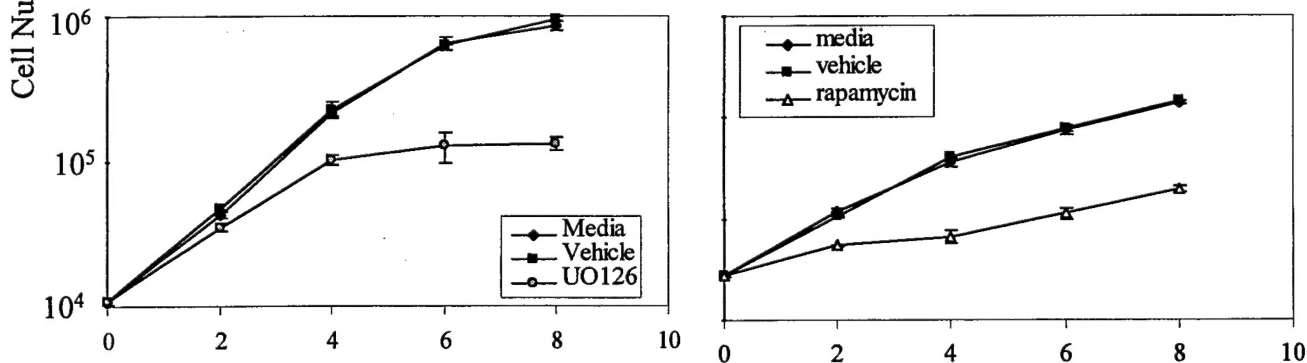
T47D:A18



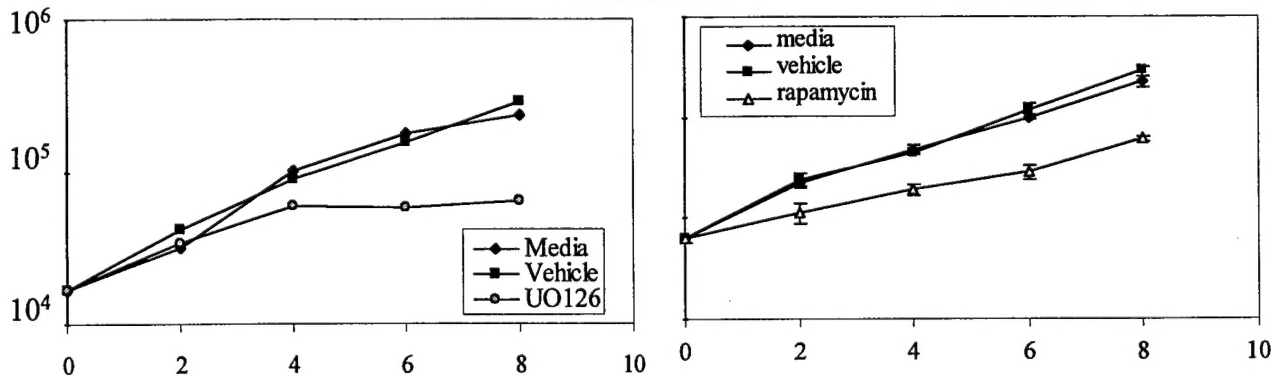
T47D:C4:2W



WWMCF-7



ECMCF-7



Day

Figure 3 (contd)

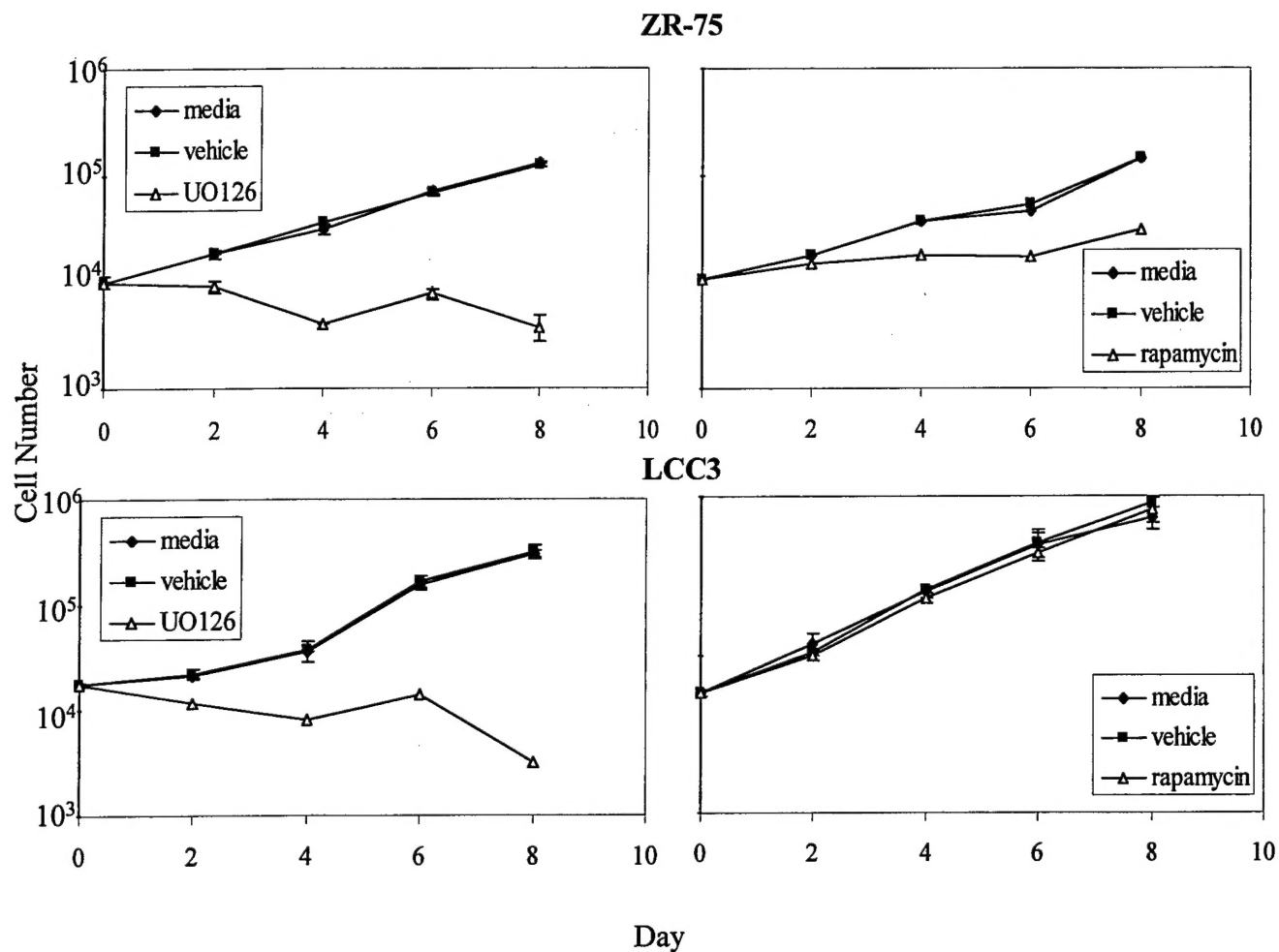


Figure 3 Growth of ER- cell is inhibited only in the presence of the MAP kinase inhibitor UO126. Panels on the left represent proliferation of breast cancer cells in normal growth media, vehicle or 10uM UO126. Cell number wa quantified from triplicate cell cultures every 48 hours. UO126 was added to media every 24 hours to a final concentration of 10uM. Panels on the right represent growth in the presence of normal growth media, vehicle or 200nM rapamycin. Cell number wa quantified from triplicate cell cultures every 48 hours. Rapamycin was added to media every 24 hours to a final concentration of 200nM. Growth of ER+ cells is inhibited in the presence of rapamycin while ER- cells maintain proliferation.

Key Research Accomplishments

- Analyzed ER+ and ER- breast cancer cell lines for expression and activation of MAP kinase
- Analyzed ER+ and ER- breast cancer cell lines for expression and activation of p70/S6 kinase
- Determined effect of p70/S6 kinase inhibition on growth of ER+ and ER- breast cancer cell lines
- Determined effect of MAP kinase inhibition on growth of ER+ and ER- breast cancer cell lines

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